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Sensitive and stable pre-calibrated solid-phase extraction columns for environmental and forensic quantification using isotope dilution mass spectrometry

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A method was developed to pre-load solid-phase extraction columns (SPE) with isotopic calibrant for use with isotope dilution mass spectrometry (IDMS). The pre-calibration method was developed, optimized, and validated for the quantification of the pesticide glyphosate in drinking water using anion-exchange SPE, electrospray-ionization, and time-of-flight mass spectrometry (ESI-TOF-MS). The instrumental method obtained a mass-accuracy of 3 ppm and a limit of quantification (LOQ) of 0.3 ng mL^{-1}. Quantification of glyphosate by IDMS significantly improved quantitative error and LOQ compared with the calibration curve. The pre-loading methodology was optimized for stability over time and validated in drinking water, exhibiting an accuracy of 1.25% ± 0.87% error with no significant difference from certified concentrations or traditional SPE. Method LOQ was 0.4 ng mL^{-1}. Quantifying glyphosate in spiked drinking water sample produced high accuracy up to two-weeks after pre-loading columns, with an accuracy of 6.41% ± 7.10% error. A potential forensic application was investigated by adapting the pre-loading method to the quantification of seven drugs in synthetic urine using a mixed-mode SPE column. The ESI-TOF-MS method using traditional SPE produced accurate quantification of all seven drugs in synthetic urine with a mean error of 4.16% ± 3.07% and LOQ of 0.780 ng mL^{-1}. The pre-loading method produced accurate quantification with 5.36% ± 4.73% error, with no significant difference from traditional SPE or certified standards. Five of the seven drugs were quantified at high accuracy one week after pre-loading, with 5.40% ± 4.57% error from certified values. This method may be applicable to analysts seeking to develop methods to improve the transfer of high-accuracy and precision methods between laboratories.

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Introduction

Implementation of high-quality mass spectrometry-based analytical methods has improved overall analytical accuracy, precision, and sensitivity attained in routine analyses.[1] Clinical and commercial laboratories have sought validated techniques to improve sensitivity, reproducibility, and accuracy through improved sample preparation, instrumental methodology, and quantitative procedures.[2,3] To achieve higher analytical quality, many laboratories have adjusted analytical emphasis from common techniques, like assays, to higher-accuracy methods, like mass spectrometric identification and quantification.[4] However, without significant training, many novel methods requiring complex sample preparation steps may not be replicated with the same quality by analysts or technicians in independent laboratories.[5] The methodological shift toward higher sensitivity and higher accuracy analytical methods[6,7] has elevated the necessity for simplified sample preparation techniques reduce the influence of sample preparation steps on final analytical quality.

Often, quantitative accuracy is negatively impacted by errors introduced by the large number of highly precise sample preparation steps required in many methods and by inherent instrumental uncertainty.[8,9] One technique used for reducing the influence of sample preparation and instrumental uncertainty on final quantitative quality is called isotope dilution mass spectrometry (IDMS).[10] IDMS is a quantification technique involving the spiking of accurate amounts of isotopically labeled analogs into an unknown sample. Using known isotopic abundances, concentration and mass of isotopically labeled spike, and mass of the unknown sample, the concentration of each analyte can be calculated mathematically without the use of calibration curves. IDMS can correct for many sources of error often associated with extraction, mass spectrometry, and

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quantification. These common sources include imprecise sample preparation, poor extraction reproducibility, sample loss, low analyte recovery, instrumental drift, matrix effects, and physical or chemical interferences. Much of the error introduced in sample preparation to change the concentration of natural analyte in a sample will affect the isotopic analog identically and be corrected in the final IDMS equation.[10,11] By reducing the influence of common error-introducing analytical steps on final quantitative quality, IDMS is capable of transferring high accuracy methods between laboratories and analysts with minimal additional training.[12] However, in-laboratory spiking of the isotopically labeled analogs into the unknown samples remains a potential source of analytical error when transferring developed methods using IDMS.

Commonly, laboratories analyzing environmental and forensic samples utilize gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), or the enzyme-linked immunosorbent assay (ELISA).[13–15] However, with increased availability, many laboratories have begun adopting sensitive and high-resolution instrumentation like time-of-flight mass spectrometers (TOF-MS) to eliminate the requirements of derivatization and chromatography.[16,17] With the advent of highly sensitive analytical methods, sample cleanup has become an important part of sample preparation to reduce harmful biological material and to pre-concentrate the analytes of interest.

Solid-phase extraction (SPE) is a sample cleanup and pre-concentration procedure to isolate analytes of interest from potentially interfering or harmful biological compounds using primarily hydrophobic interactions and cationic or anionic exchange.[18] SPE is often used on aqueous and biological samples to selectively extract and pre-concentrate analytes prior to analysis by LC/MS or electrospray ionization (ESI) TOF-MS. It was hypothesized in the research presented here that modified SPE columns could be pre-calibrated by, prior to analysis of an unknown sample, the loading of accurately known concentration of isotopically labeled analogs by highly skilled analysts. Pre-calibrated columns may be useful in transferring analytical quality between and among laboratories by removing the spiking of isotopically labeled analogs from the in-laboratory sample preparation, potentially further reducing the influence of sample preparation on final quantitative quality.

This research focused on developing a SPE pre-loading method for future implementation in improving the transfer of high accuracy and precision analytical methods between and among laboratories. For optimization experiments, the environmentally relevant pesticide glyphosate was chosen for its high usage rates, analytical difficulty, and inclusion in national drinking water regulations. Glyphosate is a polar organophosphate pesticide extensively used in the U.S. for vegetation control. The hydrophilicity and ionic character of this molecule make quantification in aqueous solution difficult. The U.S. Environmental Protection Agency (USEPA) has regulated a maximum contaminant limit of glyphosate in drinking water of 0.7 $\mu g\ mL^{-1}$.[19] Following development, optimization, and validation for the pesticide glyphosate, the potential forensic applications were explored by analysis of seven drugs of abuse in synthetic urine. For both glyphosate and the drugs of abuse, experiments were performed to assess quantitative stability over time to simulate future experiments in which pre-loaded SPE columns will be shipped to off-site laboratories for analysis.

Results

A method for the quantification of glyphosate using ESI-TOF-MS, traditional SPE, and IDMS quantification were validated by adapting and optimizing existing methods.[10,20] Validation results using IDMS quantification were compared with calibration data. Various techniques were assessed for increasing the quantitative stability over time of columns pre-loaded with glyphosate-2-^{13}C. The optimal pre-loading technique was then validated in drinking water samples spiked with unlabeled glyphosate analyzed with the pre-loaded SPE columns. Robustness of the pre-loading method was then assessed for potential forensic application by adapting and optimizing the instrumental method for seven drugs of abuse in synthetic urine. Quantitative stability over time for the SPE pre-loading method was assessed for both environmental and forensic applications.

Optimization of SPE and ESI-TOF-MS

Strata-SAX (Phenominex) columns were used in the experimental optimization of SPE elution volume and pH. It was found that the optimum efficiency of glyphosate elution occurred at pH 6.0. Therefore, an eluting solvent consisting of 1 : 1 acetonitrile/methanol acidified to 6% formic acid was used. Analysis of eluent fractions determined 16 mL was required to elute the 4 mL of 6 $\mu g\ mL^{-1}$ solution loaded onto the SPE column.

Instrumental parameters were optimized for signal intensity using negative ionization ESI-TOF-MS. Experimentally optimized instrumental parameters for drinking water samples spiked with glyphosate can be found in Table 1.

Accurate quantification was attained using isotopic distribution patterns, expected m/z shift between glyphosate and glyphosate-2-^{13}C, and mass accurate resolution of glyphosate and glyphosate-2-^{13}C.[21] For unlabeled glyphosate, a mean mass accuracy of 3.2 ppm was obtained with mean deviation of 4.3

Table 1 Experimentally optimized mass spectrometry parameters for the quantification of glyphosate

Parameter	Value
Ionization mode	Negative
Scan range	50–1000 m/z
Endplate offset	−500 V
Capillary voltage	+3750 V
Nebulizer	0.5 bar
Dry gas	4.0 L min^{-1}
Dry temperature	200 °C
Capillary exit	−100 V
Skimmer 1	−40.0 V
Hexapole 1	−23.0 V
Hexapole RF	65 Vpp
Skimmer 2	−22.0 V

ppm. For glyphosate-2-^{13}C, a mean mass accuracy was obtained of 1.7 ppm with a mean deviation of 4.1 ppm. At 168 m/z, a resolving power of 18 000 $m/\Delta m$ was obtained. Mass bias was calculated to determine the differential instrument response between the natural and isotopically labeled forms of glyphosate. A mass bias factor of 1.0006 was determined for the IDMS quantification procedure for glyphosate.[22] Optimized instrumental parameters produced the resolved spectra seen in Fig. 1. While high-resolution mass spectrometry (HR-MS) was used for mass accurate compound identification in this research on simple samples containing known compounds, it has been reported elsewhere that mass accuracy, even below 1 ppm experimental mass error, is not sufficient to accurately identify unknown analytes in samples.[23] This research optimized an instrumental method for the quantification of glyphosate in known samples only as a means of assessing the developed preloading technique and the mass accuracy of 3 ppm was therefore deemed adequate. This optimized instrumental method would not be suitable for absolute identification of samples containing unknown compounds.

Validation of SPE and ESI-TOF-MS

Instrument limit of quantification (LOQ) of ESI-TOF-MS for glyphosate in drinking water was found to be 0.312 μg mL^{-1}. Method LOQ for ESI-TOF-MS using traditional SPE and IDMS quantification for glyphosate in drinking water was found to be 0.401 ± 0.01 μg mL^{-1}. Using IDMS, quantitative accuracy and precision was maintained as concentration values were decreased approaching method LOQ, confirming previous studies on molecular IDMS quantification.[12] This LOQ sufficiently exceeded the National Primary Drinking Water Regulations from the USEPA, which specified a maximum glyphosate contaminant level of 0.700 μg mL^{-1}.

Drinking water spiked with glyphosate standard at a certified concentration of 6.00 μg mL^{-1} was analyzed using the optimized ESI-TOF-MS parameters and the traditional SPE, quantified using IDMS (n = 20). The optimized method produced an experimental concentration of 5.95 ± 0.08 μg mL^{-1}, representing an overall accuracy with 0.83% error and a precision of 1.3% RSD. These results indicate that a valid, optimized instrumental method was adapted from previously published sources to provide precise and mass-accurate quantification to be applied to the assessment of SPE pre-loading techniques.

Comparison of quantitative methodologies

Samples spiked with both labeled and unlabeled analytes at concentrations approaching the LOQ were used to create a calibration curve. Fig. 2 shows calibration data approaching the determined LOQ as well as the same data treated with IDMS quantification from the same samples, showing percent error from certified concentration, with 95% confidence intervals, at various concentrations points (n = 5 at each point). Quantitative methodologies were compared using the validated ESI-TOF-MS method with traditional SPE. Drinking water samples spiked with certified concentrations of glyphosate were quantified using IDMS with a mean accuracy of 4.22% ± 5.81% error over the concentration range 0.600–6.25 μg mL^{-1}, losing quantitative accuracy below 0.401 μg mL^{-1}. For quantification, glyphosate-2-^{13}C was spiked into all samples at 5.00 μg mL^{-1}. Calibration curve quantification lost quantitative accuracy at the 95% confidence level at 3.25 μg mL^{-1}, exhibited 59.4% ± 43.0% error the studied concentration range, but remained detectable to a concentration of 0.401 μg mL^{-1}. IDMS and calibration curve quantification demonstrated identical limits of detection, but IDMS produced a significantly lower LOQ.

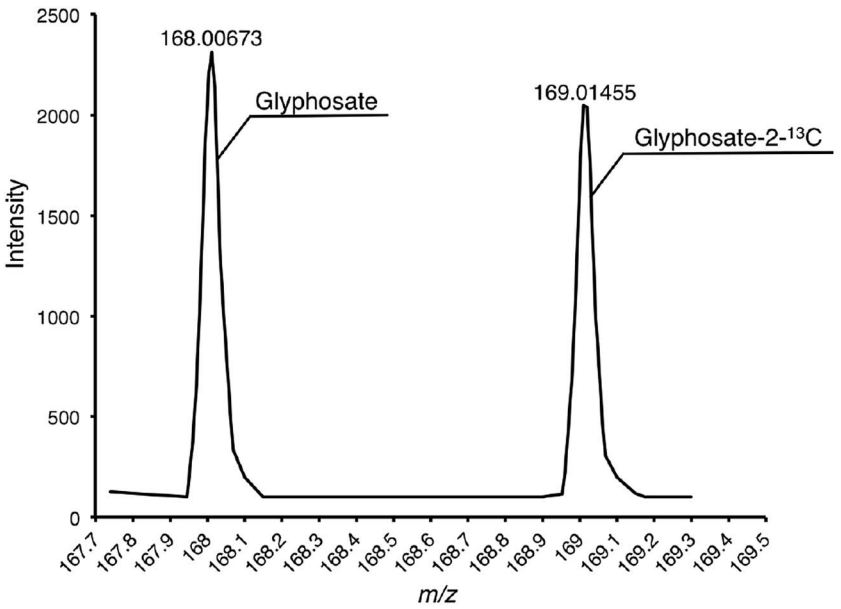

Fig. 1 Averaged mass spectra (n = 7) obtained from the analysis of drinking water spiked with 50 μg mL^{-1} of glyphosate and 50 μg mL^{-1} of glyphosate-2-^{13}C showing baseline resolution and mass accuracy.

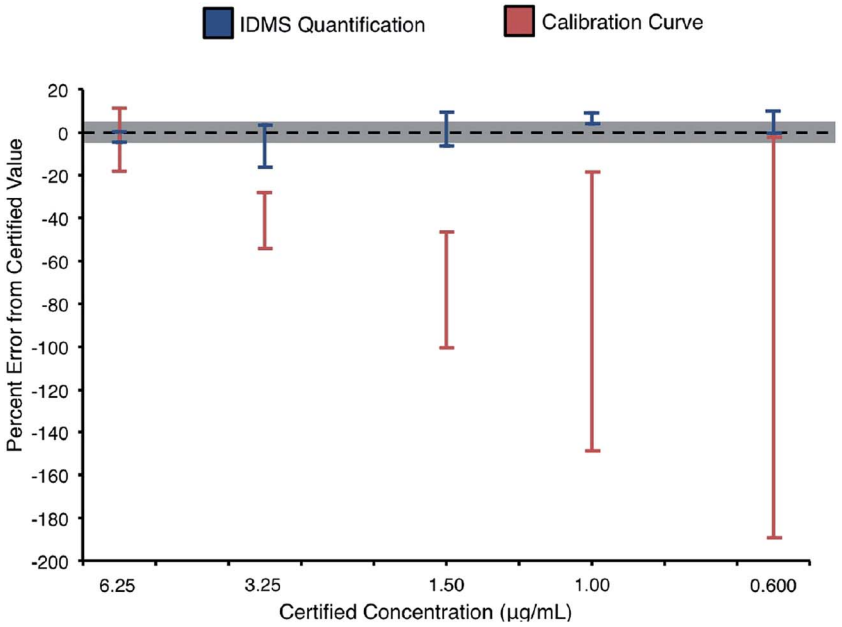

Fig. 2 A comparison of percent error of glyphosate quantification obtained in drinking water at various concentrations using IDMS and calibration curve quantifications. Dashed black line indicates certified value and shaded area represents ±5% certified error.

Optimization of pre-loading method

Samples containing glyphosate-2-^{13}C in drinking water were loaded onto the SPE columns using the manufacturer recommended procedure. These pre-loaded columns lost adequate accuracy when used for quantification of spiked drinking water samples after 1 day of storage. Several optimization procedures were then undertaken to increase on-column stability. The techniques that meaningfully impacted percent error are summarized in Fig. 3, showing absolute value of percent error using each specified method with 95% confidence intervals.

Three techniques of storing SPE columns loaded with glyphosate-2-^{13}C: air-drying columns (AD), storing with 2 mL water-rinse (WR), and storing with 2 mL methanol-rinse (MR) were assessed for quantitative stability over time. Two modifications of applying the pre-loading spikes were assessed: decreasing volume from 4 mL to 200 µL of spiking solution (DV), and spiking the solution onto an individual frit (IF) above the column packing. Combinations of storage conditions and pre-loading techniques were assessed in the optimization experiments. The optimization experiments were performed using 5 µg mL^{-1} of pre-loaded glyphosate-2-^{13}C and 5 µg mL^{-1} of glyphosate ($n = 5$ for each technique).

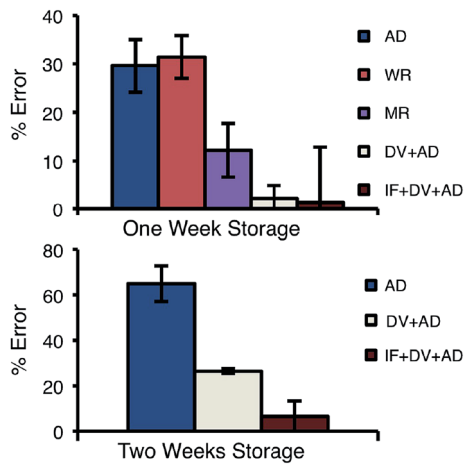

Fig. 3 The effects on absolute value of percent error of multiple optimization techniques after (top) one week of storage and (bottom) two weeks of storage, with 95% confidence intervals shown.

At one week of storage, AD and WR showed no statistical difference in percent error, but MR exhibited statistically decreased percent error from both AD and WR ($p < 0.05$). However, both WR and MR produced disruption of the SPE column packing material, which lead to irreversible binding of glyphosate-2-^{13}C. Irreversible binding of the pre-loaded isotope was suspected in WR and MR by the production of large positive bias after storage for two weeks. Irreversible binding was confirmed by the observation of large negative bias by pre-loading unlabeled analytes and quantifying a solution of isotopically labeled analytes after two weeks of storage. Therefore, WR and MR were eliminated from the optimization experiments. The modified pre-loading procedure of DV + AD significantly reduced percent error compared with AD, WR, and MR (all $p < 0.05$). The modified pre-loading procedure of IF + DV + AD produced significantly decreased percent error compared with AD and WR (all $p < 0.05$).

After two weeks of storage, all optimization techniques increased in percent error compared with the same technique after one week. DV + AD exhibited significantly lower percent error compared with AD after two weeks of storage ($p < 0.05$), but did not demonstrate adequate quantitative accuracy (26.6% ± 1.12% error). IF + DV + AD exhibited further significant decrease in percent error compared with DV + AD ($p < 0.05$) and maintained adequate quantitative accuracy (6.41% ± 7.14% error).

When extended to four weeks of storage, no technique or combination of techniques yielded adequate quantitative accuracy, with IF + DV + AD exhibiting 47.2% ± 1.35% error. Results for IF + DV + AD over four weeks can be found in Fig. 4. Significant loss of stability was observed between two and four weeks for pre-loaded columns. It was determined that the combination IF + DV + AD would be used in the pre-loading method to maintain high-quality quantification up to two weeks after pre-loading. Future research will work to improve the stability of on-column analyte stability during storage.

Validation of pre-loading technique

Using the validated instrumental parameters from the optimized ESI-TOF-MS method and the IF + DV + AD technique optimized for SPE pre-loading, a validation experiment was performed on drinking water spiked with 6 μg mL^{-1} of glyphosate ($n = 20$). Columns were pre-loaded with isotopically labeled glyphosate at a concentration of 6 μg mL^{-1} 30 minutes prior to analysis of the spiked drinking water to allow the columns time to air-dry. Drinking water samples analyzed 30 minutes after pre-loading columns with isotopes produced a final concentration of 5.93 ± 0.052 μg mL^{-1}, representing a 1.25% error and a 0.87% RSD. A method LOQ of 0.401 μg mL^{-1} was obtained for the pre-loaded columns, identical to the LOQ obtained for ESI-TOF-MS using traditional SPE. These results indicate that the IF + DV + AD pre-loading technique produced valid quantitative results that did not differ significantly from either the certified value or quantitative results from the ESI-TOF-MS method using traditional SPE at the 95% confidence level.

Potential application to drugs of abuse in synthetic urine

The optimized and validated ESI-TOF-MS analytical method was optimized for the quantification in synthetic urine of seven common opioids and alkaloids used as adulterants. Experimentally optimized instrumental parameters can be found in Table 2. Analysis of these opioid and alkaloid compounds required changing the SPE column from the anion-exchange column used for glyphosate to a Clean Screen DAU (UCT) mixed-mode hydrophobic and cation-exchange column.

The optimized instrumental parameters produced valid quantification for all seven drugs spiked in synthetic urine at 40 ng mL^{-1} following traditional SPE extraction. Validation results for ESI-TOF-MS using traditional SPE and IDMS can be found in Table 3 comparing certified concentrations with experimentally determined concentrations with 95% confidence intervals. All drugs of abuse were quantified with <10% quantitative error and <20% RSD. Accuracy ranged from 0.689% to 9.33% error for methadone and cocaine, respectively. Precision ranged from 0.973% to 11.6% RSD for fentanyl and morphine, respectively. Mean quantitative accuracy for samples analyzed using ESI-TOF-MS with traditional SPE exhibited mean accuracy of 4.16% ± 3.07% error and mean precisions of 5.93% ± 3.03% RSD. Experimentally determined concentrations did not differ significantly from certified concentrations at the 95% confidence level.

The method LOQ determined for the drugs of abuse using the optimized ESI-TOF-MS with traditional SPE and IDMS of 0.780 ng mL^{-1} for all analytes. As with the work with glyphosate, quantification with IDMS produced significantly higher accuracy and precision approaching the LOQ. In Fig. 5, IDMS and typical calibration curve quantitation are compared in the same samples using the validated ESI-TOF-MS method and traditional SPE, showing mean percent difference from certified concentrations of all analytes included in this study at various concentration points, with 95% confidence ranges ($n = 5$). IDMS exhibited a mean percent error across all concentration points for all analytes of 5.66% ± 10.9%. Calibration curve lost quantitative accuracy below 3.13 ng mL^{-1}, exhibiting a mean percent error for the concentrations range 25.0 ng mL^{-1} through 3.13 ng mL^{-1} of 11.5% ± 27.8%.

The IF + DV + AD pre-loading technique was validated for the drugs of abuse using the mixed-mode SPE column, analyzed by the optimized and validated ESI-TOF-MS instrumental method. Analysis of synthetic urine spiked with 40 ng mL^{-1} of all seven drugs of abuse using pre-loaded SPE columns 30 minutes after

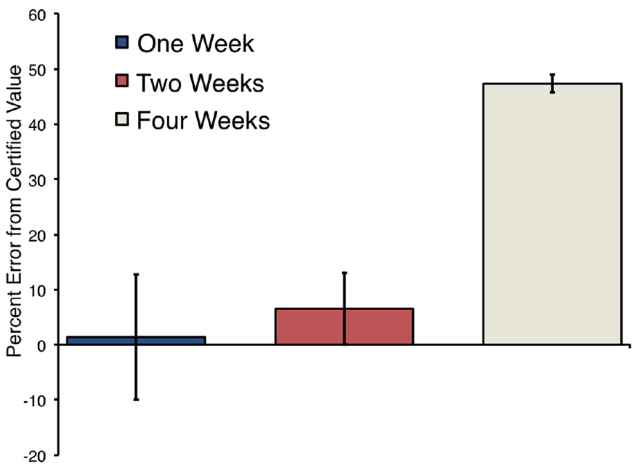

Fig. 4 Quantitative stability over time of SPE columns pre-loaded with isotopically labeled glyphosate. Percent error and 95% confidence errors are shown.

Table 2 Experimentally optimized mass spectrometry parameters for the quantification of opioid and alkaloid drugs of abuse

Parameter	Value
Ionization mode	Positive
Scan range	240–400 *m/z*
Endplate offset	−500 V
Capillary voltage	−4500 V
Nebulizer	0.4 bar
Dry gas	4.0 L min^{-1}
Dry temperature	200 °C
Capillary exit	135 V
Skimmer 1	40.0 V
Hexapole 1	23.0 V
Hexapole RF	250 Vpp
Skimmer 2	24.0 V

Table 3 Validation values for opioids and alkaloids using ESI-TOF-MS, traditional SPE, and IDMS quantification for drugs of abuse in synthetic urine, showing 95% confidence

SPE-ESI-TOF-MS ($n = 5$)	Calculated value (ng mL^{-1})	Experimental value (ng mL^{-1})	|% error|	% RSD	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Morphine	40 ± 2.00	42.2 ± 6.65	5.57	11.6	0.446	0.800
Codeine	40 ± 2.00	42.3 ± 4.44	5.86	7.73	0.202	0.800
Cocaine	40 ± 2.00	43.7 ± 2.18	9.33	3.69	0.773	0.800
Methadone	40 ± 2.00	39.7 ± 2.32	0.689	4.32	0.128	0.800
6-AM	40 ± 2.00	40.3 ± 0.531	0.797	6.12	0.0502	0.800
Fentanyl	40 ± 2.00	42.4 ± 4.10	0.710	0.973	0.234	0.800
Heroin	40 ± 2.00	42.2 ± 6.65	6.18	7.13	0.238	0.800

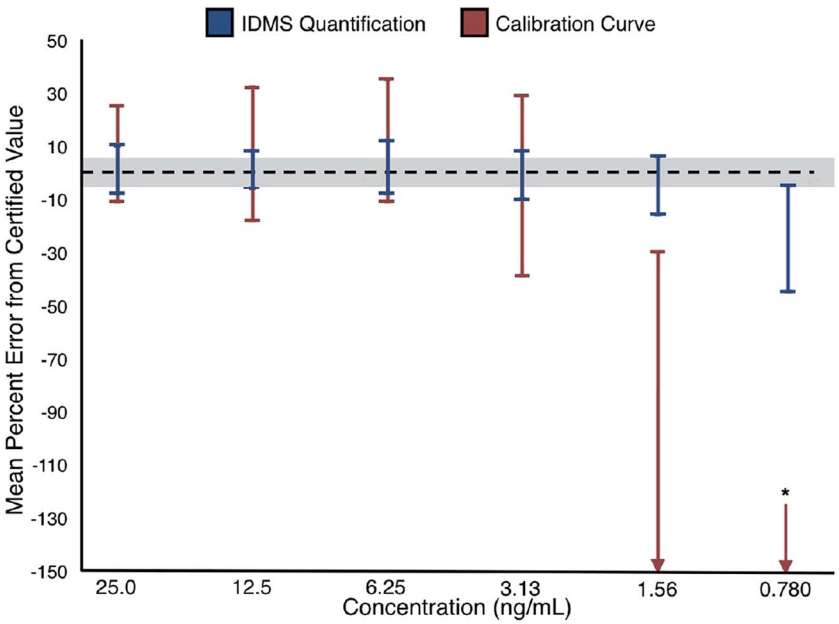

Fig. 5 A comparison of mean accuracy and precision (95% confidence range) of experimental values obtained in synthetic urine with calibration curve and IDMS across all listed analytes. Dashed black line indicates certified value and shaded area representing ±5% certified error. * Confidence interval falls outside of viewable range.

pre-loading produced highly accurate quantitative results. All opioids and alkaloids with the exception of cocaine were quantified with ≤10% quantitative error and <20% RSD. Accuracy ranged from 0.766% to 13.0% error for codeine and cocaine, respectively. A mean accuracy of 5.36% ± 4.73% error and mean precision of 6.58% ± 2.92% RSD were produced by the pre-loading method. The mean experimental values obtained did not differ from the mean certified values or from the concentrations obtained using the validated ESI-TOF-MS and traditional SPE method at the 95% confidence level. Validation results for the quantification of all seven opioids and alkaloids spiked in synthetic urine and extracted using pre-loaded SPE columns 30 minutes after pre-loading can be found in Table 4.

Table 4 Validation values for opioids and alkaloids using ESI-TOF-MS, SPE pre-loading method, and IDMS quantification in synthetic urine, showing 95% confidence

Pre-loading ($n = 5$)	Calculated value (ng mL^{-1})	Experimental value (ng mL^{-1})	|% error|	% RSD	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Morphine	40 ± 2.00	40.6 ± 1.61	1.59	2.93	0.429	0.8
Codeine	40 ± 2.00	39.7 ± 3.40	0.766	6.32	0.214	0.8
Cocaine	40 ± 2.00	34.8 ± 5.07	13.0	10.1	0.737	0.8
Methadone	40 ± 2.00	44.0 ± 6.23	10.0	10.4	0.156	0.8
6-AM	40 ± 2.00	39.4 ± 1.87	1.56	3.43	0.0552	0.8
Fentanyl	40 ± 2.00	40.3 ± 4.49	0.873	8.21	0.222	0.8
Heroin	40 ± 2.00	36.2 ± 1.94	9.52	3.95	0.223	0.8

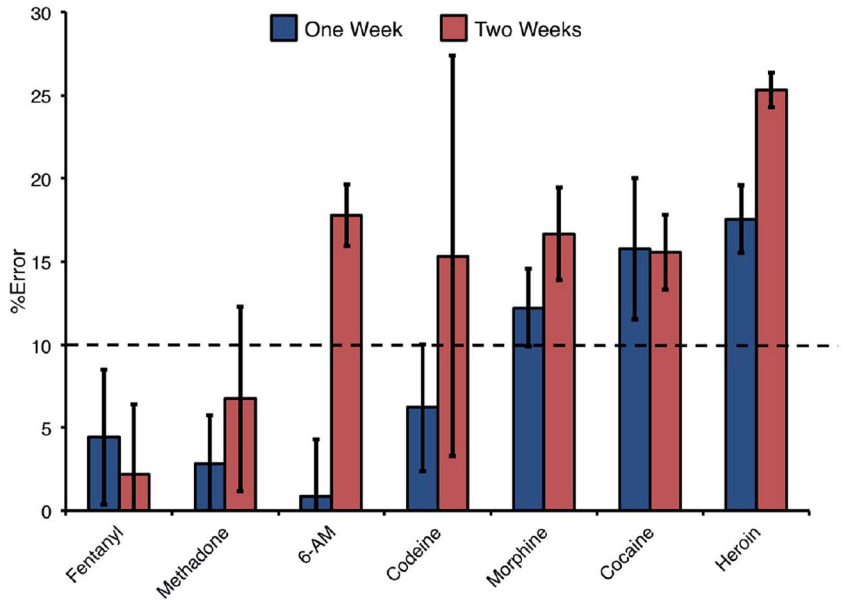

Fig. 6 Mean % error of pre-loaded columns after storage for indicated amounts of time, showing 95% confidence intervals. Dashed line represents the 10% error from certified value defined as adequate accuracy in this study.

Using the ESI-TOF-MS instrumental method and the IF + DV + AD pre-loading method, stability over time was assessed for the analysis of synthetic urine samples spiked with the drugs of interest. Morphine, codeine, methadone, 6-acetylmorphine, and fentanyl were quantified with adequate accuracy when analyzed one week after column pre-loading. Methadone, fentanyl, and codeine maintained adequate quantitative accuracy when quantified two weeks after column loading. At the two-week time interval, codeine maintained accuracy at the 95% confidence interval, but produced poor precision (±12%). Cocaine and heroin were not quantified accurately after one week (15%, 17% error, respectively) or after two weeks (15%, 25% error, respectively) of storage. The stability over time results are summarized in Fig. 6.

The work presented here demonstrates the viability of isotopically pre-loaded SPE columns for the quantification of environmentally and forensically relevant analytes by IDMS. This viability was demonstrated with different SPE column packing types and a wide range of investigated analytes. While the pre-loading technique produced stable quantitative accuracy over time for 70% of the chosen drugs of abuse, the concept of pre-loading and storing SPE columns for future analysis has been proven viable by this research. Future work will increase the breadth and robustness of this developed method. In subsequent research, the transfer of analytical quality using this pre-loading method will be tested in by shipment of pre-loaded SPE columns to independent laboratories for quantification of determined analytes. Providing analysts with pre-calibrated, pre-loaded SPE columns to be used for IDMS quantification may help to improve overall analytical quality.

A limitation of this research is the potential for introduction of matrix-effect errors into quantitative analysis. Traditional IDMS eliminates errors introduced by matrix-effects by equilibrating isotopic and natural molecules in a native solution prior to all other sample preparation. Any matrix effects altering the concentration of a free molecule of interest will equally affect the isotopic analog, without altering the isotope-to-natural ratio within the sample. Using the process described in this work, equilibration between the isotopic and naturally occurring molecules of interest will not occur until after elution from the SPE column. Equilibration at this post-elution step allows IDMS to correct for analytical errors typically introduced in instrumentation, but will not correct for matrix effects that have already altered the concentration of naturally occurring molecule in the sample. This research used only drinking water samples and synthetic urine samples, both relatively simple matrices. As high accuracy and precision were obtained in method validation, the post-elution equilibration was determined to have little effect on analytical quality. However, as the isotopes were pre-loaded in a relatively simple sample matrix (drinking water), future research will focus on the analysis of unknown water and urine samples containing complex matrixes to investigate potential effects on quantitative accuracy.

Experimental

Chemicals and standards

Glyphosate (99% pure) and glyphosate-2-^{13}C (99% pure, 99% enriched) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Drinking water samples were supplied by Pittsburgh Municipal Water (Pittsburgh, PA, USA). Heroin, 6-AM, morphine, cocaine, methadone, and fentanyl analytical standards at certified concentrations of 1.0 mg mL^{-1} and codeine analytical standard at a certified concentration of 100 μg mL^{-1} were purchased from Cerilliant (Round Rock, TX, USA). The deuterium-enriched analogs: heroin-D_9, morphine-D_3, cocaine-D_3, codeine-D_3, methadone-D_3, and fentanyl-D_5 at certified

concentrations of 100 μg mL^{-1} and 6-AM-D_3 at a certified concentration of 1.0 mg mL^{-1} were purchased from Cerilliant. Synthetic urine, HPLC grade methanol, HPLC grade water, hyclone phosphate buffered saline (PBS), HPLC grade 2-propanol, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetate buffer was prepared using sodium acetate and acetic acid purchased from Fisher Scientific. Each naturally occurring analyte was prepared in separate stock solutions at 10 μg mL^{-1} for the drugs of abuse and 60 μg mL^{-1} for glyphosate in HPLC grade water. For glyphosate, two solutions were prepared by mass in drinking water: one spiked with 6 μg mL^{-1} of glyphosate and one spiked with 6 μg mL^{-1} of glyphosate-2-^{13}C. Two solutions were prepared by mass in synthetic urine for the drugs: one containing all unlabeled drugs at 40 ng mL^{-1} and one containing all isotopically labeled analogs at 40 ng mL^{-1}.

Solid-phase extraction

For glyphosate experiments, Strata-SAX SPE columns (500 mg bed mass, 6 mL volume) were purchased from Phenomenex (Torrance, CA, USA) for the analysis of glyphosate in drinking water. Columns were conditioned per manufacture recommendations with 4.0 mL HPLC grade methanol and 4.0 mL HPLC grade water. Then, 4.0 mL of drinking water sample was loaded onto the column and washed with 4.0 mL of HPLC grade methanol. Elution was performed with 16 mL of a 1 : 1 solution of acetonitrile and methanol acidified at 6% with formic acid. All SPE analyses were performed with a negative pressure vacuum chamber at 1 mL min^{-1}. For IDMS quantification, a solution of glyphosate-2-^{13}C in HPLC grade water was loaded onto the column after conditioning but before the spiked drinking water sample was loaded.

For the drugs of abuse, CSDAU303 (UCT, Bristol, PA) SPE were used for extraction of drugs from synthetic urine. Columns were conditioned with 2.0 mL of HPLC methanol, 2.0 mL HPLC water, and 2.0 mL PBS. Extraction was performed on 4.0 mL synthetic urine spiked with unlabeled analytes, followed by a wash of 4.0 mL water, 3.0 mL acetate buffer, and 3.0 mL of methanol. For IDMS experiments, a 4.0 mL aliquot of the isotopically enriched working solution (phosphate-buffered to pH 6) in synthetic urine was loaded onto the column next. After a two minute on-column drying period, all extracted compounds were eluted from the column using 11.0 mL ethyl acetate : 2-propanol : ammonium hydroxide (84 : 12 : 1) solution.

ESI-TOF-MS

A Bruker Daltonics microTOF (Billerica, MA, USA) mass spectrometer with an orthogonal ESI source was experimentally optimized for the analysis of all analytes of interest and their deuterium-enriched analogues. Samples were infused using the ESI source at a flow rate of 240 μL $hour^{-1}$ with a Cole Palmer 74900-00 syringe pump (Vernon Hills, IL). The experimentally optimized method parameters can be found in the Results section. Quantitative *m*/*z* ions were experimentally found for each labeled and unlabeled analyte. Quantitative ions for each natural and isotopic compound can be found in Table 5.

Table 5 Quantitative ions for each studied analyte and the corresponding exact and measured masses

Analyte	Quantitative ion	Exact mass (*m*/*z*)	Measured mass (*m*/*z*)
Glyphosate	$C_3H_7NO_5P^-$	168.00618	168.00673
Morphine	$C_{17}H_{20}NO_3^+$	286.14432	286.15051
Codeine	$C_{18}H_{22}NO_3^+$	300.15997	300.16225
Cocaine	$C_{17}H_{22}NO_4^+$	304.15488	304.15816
Methadone	$C_{21}H_{28}NO^+$	310.21709	310.22002
6-AM	$C_{19}H_{22}NO_4^+$	328.15488	328.15521
Fentanyl	$C_{22}H_{29}N_2O^+$	337.22799	337.2277
Heroin	$C_{21}H_{24}NO_5^+$	370.16545	370.16887
Glyphosate-2-^{13}C	$C_2{}^{13}C_1H_7NO_5P^-$	169.01485	169.01455
Morphine-D_3	$C_{17}H_{17}D_3NO_3^+$	289.17031	289.1708
Codein-D_3	$C_{18}H_{19}D_3NO_3^+$	303.18596	303.18769
Cocaine-D_3	$C_{17}H_{19}D_3NO_4^+$	307.18088	307.18249
Methadone-D_3	$C_{21}H_{25}D_3NO^+$	313.24308	313.24389
6-AM-D_3	$C_{19}H_{19}D_3NO_4^+$	331.18088	331.1833
Fentanyl-D_5	$C_{22}H_{24}D_5N_2O^+$	342.27131	342.27417
Heroin-D_9	$C_{21}H_{15}D_9NO_5^+$	379.24343	379.24523

SPE pre-loading

SPE column pre-loading followed the same conditioning and washing procedures for the separate columns listed in the Solid-phase extraction section. For pre-loading, matrix-matched solutions containing isotopic analogs were extracted and allowed to air-dry for a determined period prior to extraction of solutions spiked with certified concentrations of unlabeled analytes.

Optimization of pre-loading procedure assessed three methods of storing columns: allowing columns to air-dry (AD) in an upright position, rinsing the columns with 2 mL of HPLC grade water (WR) and storing wet with Parafilm sealing both ends of the column, and rinsing the columns with 2 mL of HPLC grade methanol (MR) and sealing with Parafilm. Two sample extraction modifications were assessed: decreasing the volume (DV) of the isotopically labeled solution from 4 mL to 200 μL, and placing an individual frit (IF) approximately 2 cm above the column packing. Assessment of these techniques followed a reasonable order. The storage methods were testing first and comparisons were generated between all three. The optimal method that provided the greatest long-term quantitative stability was used for all future analyses. Pre-loaded columns were cold-stored at 15 °C for one week, two weeks, and four weeks prior to analysis of a solution of drinking water spiked with certified concentrations of glyphosate. Five replicates we performed for each technique at each time interval. Prior to ESI-TOF analysis, all eluted mixtures of naturally occurring and isotopically labeled molecules were vortexed for 5 minutes to allow time for equilibration.

Method validations

For validation experiments, solutions were prepared by spiking certified standards for each analyte being studied into either drinking water or synthetic urine matrix by mass. The appropriate sample preparation was applied (either SPE using the

above method or SPE pre-loading using the above method) and the eluate was analyzed using the above method for ESI-TOF-MS. Quantification was performed by IDMS. Replicates were performed for all validation experiments ($n = 20$ for glyphosate by SPE and SPE pre-loading, $n = 5$ for drugs by SPE and SPE pre-loading). Experimentally obtained values were compared with calculated values at the 95% confidence level. For assessment of accuracy, an adequate percent error to be achieved by the method being validated was set at ±10%.

Quantification methods

Quantification was accomplished using IDMS, as defined in EPA Method 6800.[10] The concentration of the unknown natural molecule was calculated as:

$$\text{Concentration } (\mu\text{mol g}^{-1}) = [(C_s W_s/W_x) \times {}^{i}P_s - (R_{i/n} \times {}^{n}P_s)]/[(R_{i/n} \times {}^{n}P_x) - {}^{i}P_x],$$
$$R_{i/n} = \text{signal intensity of enriched molecule/signal intensity natural molecule} \quad (1)$$

In the IDMS quantitation procedure, C_s and C_x are the concentrations, in μmol g^{-1}, of the selected analyte in the isotope-enriched spike and the spiked sample, respectively. ${}^{i}P_s$ and ${}^{i}P_x$ are the percent purity of the isotopically enriched molecule in the spike and the pre-spiked sample, respectively. Likewise, ${}^{n}P_s$ and ${}^{n}P_x$ are the percent purity of the naturally occurring analyte in the spike and the pre-spiked sample, respectively. $R_{i/n}$ is the ratio of peak areas of the quantitative ion for the natural and isotopically enriched form of the compound being analyzed obtained in the same analysis. Finally, W_s and W_x are the masses of the spike and sample, respectively. Peak areas corresponding to the pair of natural and isotopically enriched analytes were exported to an in-house spreadsheet for IDMS quantitation.

For comparison of quantification methodologies, solutions were prepared in the appropriate matrix, drinking water or synthetic urine, at various concentrations. For glyphosate, drinking water samples were prepared containing unlabeled glyphosate at the concentrations 6.25, 3.25, 1.50, 1.00, and 0.600 μg mL^{-1}. At each concentration point, an equal concentration of isotopically labeled glyphosate was used for IDMS. For the drugs, synthetic urine samples were prepared containing unlabeled versions of each analyte six concentration points: 25.0, 12.5, 6.25, 3.13, 1.56, and 0.780 ng mL^{-1}.

For all IDMS quantification during method development, method validation, and application, isotopically labeled analogs of all analytes of interest were spiked into samples by volume to a precisely known concentration. Glyphosate-2-^{13}C was spiked into all samples at 5.00 μg mL^{-1} and all illicit drugs at 40.0 ng mL^{-1}.

Mass bias factor

A mass bias factor was determined experimentally to mathematically correct for the differences in signal intensity between the natural and isotopic forms of an analyte in samples at identical concentrations. Samples of 4.0 mL of the mixture of working solutions containing both natural and isotopically enriched analytes at 20.0 ng mL^{-1} were analyzed ($n = 28$) to determine mean signal intensities for each natural and each isotopic analyte. A ratio was computed representing the signal intensity of a natural compound as a function of the signal intensity of the isotopic analog at identical concentrations.[22] This mass bias factor was applied as a correction factor to the isotope signals in the IDMS equation, as given by the following equation:[10]

$$\text{Concentration } (\mu\text{mol g}^{-1}) = \text{mass bias} \times [(C_s W_s/W_x) \times {}^{i}P_s - (R_{i/n} \times {}^{n}P_s)]/[(R_{i/n} \times {}^{n}P_x) - {}^{i}P_x],$$
$$\text{mass bias} = A_N C_i / A_i C_N \quad (2)$$

where A_N and A_i are the signal obtained from the TOF-MS for the natural analyte and the isotopic analog, respectively. C_N and C_i are the concentrations of the natural analyte and isotopic analog, respectively. Mass bias factors were computed for each compound and used as an internal correction factor in the quantitative method.

Statistics

For measurements of accuracy and precision, the percent error from calculated concentration (% error), percent relative standard deviation (% RSD), and 95% confidence intervals were used. For validation comparisons, the 95% confidence interval of the experimentally determined mean was compared with the accompanying ±5% standard deviation of the certified standard. For comparison of means, p-values were computed to compare the statistical similarity of two means with unequal variance, with $p < 0.05$ indicating statistically significant difference. All analyses in synthetic urine were blank-subtracted using synthetic urine samples that were not spiked with either the natural or isotopically enriched analytes. Analyte-specific limit of detection (LOD) was calculated using replicate measurements ($n = 10$) of a method blank multiplied by three-times the standard deviation of the blank measured at expected measured mass of the compounds determined in method development.[24] Limit of quantification (LOQ) was determined as the concentration at which the experimentally determined concentration ($n = 10$) of a compound differs to a statistically significant degree from the calculated concentration of the compound at the 95% confidence level. Essentially, LOQ was established to be the concentration at which quantitative accuracy was lost at the 95% confidence level.

Conclusions

This research has demonstrated viability of modifying SPE columns to produce pre-calibration by loading columns with accurately known concentrations of isotopically labeled analogs to remain quantitatively stable over a necessary amount of time. The method for column pre-loading was developed, optimized, and validated for quantification of the organophosphate pesticide glyphosate in drinking water. This pre-loading method was then adapted and applied to a different column packing material for the quantification of seven drugs of abuse in synthetic

urine. The pre-loading method was shown to be highly quantitatively stable over a period of two weeks for glyphosate and one week for all drugs of abuse, excluding cocaine and heroin. This work is useful to researchers seeking to develop methods to improve the transfer of high accuracy and precision analytical methods between and among laboratories. It was the objective of this research to reduce the complexity of sample analysis by producing a method without derivatization, chromatography, or calibration curves. As such, this work did not explore the application of pre-loaded SPE columns to the many forms of instrumental analysis for which IDMS has been previously successfully performed, including gas and liquid chromatography-mass spectrometry. Future work will expand the method validation to include a range of analytical instrumentation. However, as IDMS requires mass analysis, the method validated by this work is incompatible with instrumentation utilizing measurement techniques that do not measure ion mass, such as electromagnetic spectroscopy (*e.g.* fluorometry, atomic absorption/emission spectroscopy, infrared spectroscopy) and electroanalytical methods (*e.g.* amperometry, coulometry, potentiometry). Future work will be conducted on further increasing the on-column stability of analytes, increasing the breadth of applicable analytes and columns, and evaluation of field-deployment into off-site laboratories.

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